INFECTION AND IMMUNITY, Feb. 1982, p. 738–740
0019-9567/82/020738-03$02.00/0

Vol. 35, No. 2

Non-Immunoglobulin Fraction of Human Milk Protects Rabbits Against Enterotoxin-Induced Intestinal Fluid Secretion

ANNE-BRIT OTNAESS[1]* AND ANN-MARI SVENNERHOLM[2]

Pediatric Department and Institute for Experimental Medical Research, Ullevaal Hospital, Oslo 1, Norway,[1] and Institute of Medical Microbiology, University of Gøteborg, Gøteborg, Sweden.[2]

Received 6 July 1981/Accepted 25 September 1981

Human milk was fractionated by ammonium sulphate precipitation and column chromatography. A milk fraction depleted of secretory immunoglobulin A and with an apparent molecular weight of greater than 400,000 inhibited fluid secretion induced by cholera toxin and *Escherichia coli* heat-labile toxin in rabbit ileal loops.

Human milk may protect children from gastrointestinal infections (2), and specific antibodies against agents that may cause gastroenteritis have been demonstrated in milk (4). In addition, milk has been found to contain non-immunoglobulin components which may be of importance for the prevention of infections in infants (3).

Recently, we have described a high-molecular-weight, non-immunoglobulin fraction of human milk which inhibited *Escherichia coli* heat-labile toxin (LT) (5), which is known to cause gastroenteritis in children and adults (8). The inhibitory activity of the milk was demonstrated by the in vitro immunological test enzyme-linked immunosorbent assay (ELISA).

In this report, we have studied the capacity of milk and various secretory immunoglobulin A (IgA) and non-secretory IgA-containing milk fractions to inhibit fluid secretion induced by LT or cholera toxin (CT) in rabbit small bowel loops. A protective effect of milk secretory IgA but also of a fraction without detectable immunoglobulin was demonstrated.

Milk from a healthy Norwegian woman was depleted of fat and cells by centrifugation (40,000 × *g*, 2 h). Ammonium sulphate was added to 50% saturation to precipitate immunoglobulins, and the precipitated and non-precipitated fractions were dialyzed against phosphate-buffered saline (PBS), pH 7.4. After concentration, samples of the fractions were submitted to column chromatography on an AcA44 column (LKB, Stockholm, Sweden) (6). The content of secretory IgA was measured by rocket immunoelectrophoresis, using purified secretory IgA from human milk as standard (6), and IgG and IgM were quantitated by single radial immunodiffusion. The enterotoxin inhibitory activity of the various milk fractions was measured by ELISA as previously described (5) and in rabbit small bowel loops (9).

Briefly, the ELISA was performed in polyvinyl microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) coated with 1:10,000 diluted antiserum to purified CT prepared in burro (kindly provided by J. B. Robbins, Bureau of Biologics, Bethesda, Md.). The plates were incubated for 1 h at 37°C with (i) the toxin sample preincubated for 30 min at room temperature with milk fraction or buffer, (ii) anti-CT prepared in rabbit, and (iii) anti-rabbit IgG prepared in swine (Orion, Finland) and coupled to alkaline phosphatase. The substrate disodium para-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) was added, and the reaction was stopped after 30 to 60 min by the addition of 2 N NaOH. The optical density at 405 nm was read in a Multiscan (Titertek; Irvine, Scotland). The crude LT stock solution (1.8 g/liter) was diluted to 0.2 g/liter to give an optical density at 405 mm of 0.8 to 1.4 in the ELISA, (100% toxin). Upon further dilution, a linear standard curve was obtained. The inhibitory activity of milk fractions is expressed as the percent inhibition of the binding of LT to the ELISA plate. The antisera against CT neutralized both CT and LT when tested in an adrenal cell assay (1).

In the in vivo experiments with rabbit small bowel loops (9), four dilutions of LT or CT were mixed with each milk fraction or with PBS. Each sample was tested in various positions in at least three different rabbits. Crude LT or purified CT (Schwarz-Mann, Orangeburg, N.Y.) was used. After incubation of toxin and milk for 30 min at room temperature, the mixtures were injected

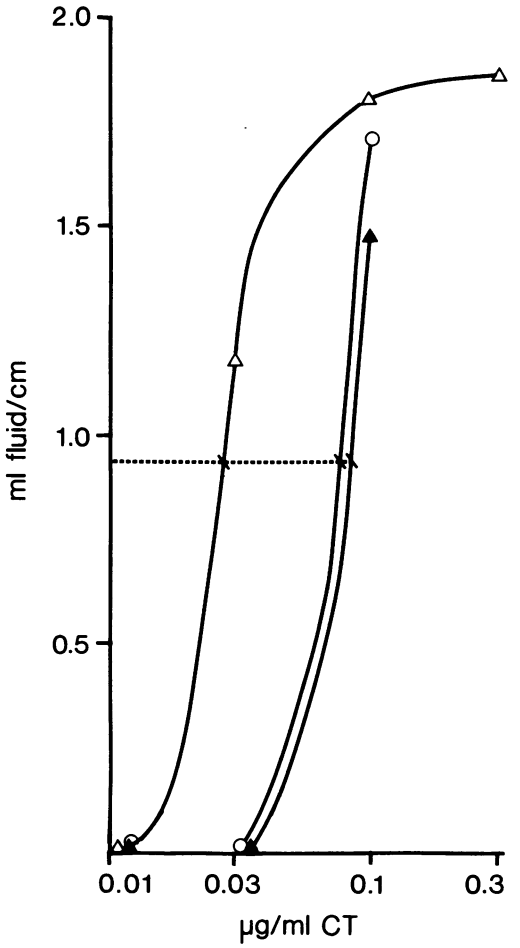

FIG. 1. Fluid accumulation in rabbit ileal loops 18 h after the injection of graded doses of CT with milk fractions or buffer. The fluid accumulation is expressed as milliliters of fluid per centimeter of loop., Symbols: △, CT; ○, CT plus peak 1a (0.014 mg/ml); ▲, CT plus fraction B (3.2 mg/ml).

into 5-cm-long small bowel loops along the entire small intestine of each rabbit as described (9). The fluid accumulation and the length of the loops were measured after 18 h. The 50% effective dose ED_{50} was determined as the toxin concentration giving 50% of maximal fluid accumulation in the loops, and the protection efficacy was calculated as the ratio between the ED_{50} for toxin with a milk fraction and the ED_{50} for toxin with PBS.

In each rabbit, a dose response curve for the toxin in PBS was prepared. When some of the milk fractions were introduced into the small bowel loops together with the toxin, a reduced fluid accumulation was observed (Fig. 1). An increased protection was obtained when a higher

TABLE 1. Effect of milk fractions on enterotoxin in vivo and in vitro

Milk fraction	IgA % of total protein	In vivo (rabbit ileal loops)				In vitro (ELISA)
		Protection efficacy[a]		Protection efficacy per mg of milk protein		% Inhibition[b] of LT binding
		CT	LT	CT	LT	
Fraction A (sediment after ammonium sulphate treatment)	14	2	4	0.3	0.7	30
Fraction A after gel filtration, peak I (MW,[c] >400,000)	95	16	9	4.5	2.8	60
Fraction A after gel filtration, peak II (MW, 70 to 200,000)	0	NT[d]	1	—[e]	—	0
Fraction A after gel filtration, peak III (MW, <40,000)	0	NT	1	—	—	0
Fraction B (supernatant after ammonium sulphate treatment)	3	19	25	0.6	0.8	40
Fraction B after gel filtration, peak 1a (MW, >400,000)	0	18	49	130	160	100
Fraction B after gel filtration, peak 1b (MW, 400,000)	10	1	1	—	—	0
Fraction B after gel filtration, peak 2 (MW, 70 to 200,000)	0	1	1	—	—	0

[a] Protection efficacy = $[(ED_{50}$ toxin + milk fraction)/$(ED_{50}$ toxin + PBS)]
[b] Final concentration, 0.025 mg of milk protein per ml in the test well.
[c] MW, Molecular weight.
[d] NT, Not tested.
[e] —, Definition.

concentration of active milk inhibitory fraction was used.

The peaks obtained after gel filtration (Table 1) were tested at a final protein concentration of 0.16 to 0.40 mg/ml, except peak 1a, which was diluted 10-fold further (0.014 mg/ml). Fractions A and B were tested at 3 to 4.5 mg of protein per ml. Some inhibitory effect was found for peak I after gel filtration of the ammonium sulphate-precipitated fraction (fraction A, Table 1), which consisted of 95% IgA. However, considerably more inhibitory activity per milligram of protein was eluted in a fraction (peak 1a) after column chromatography of fraction B (Table 1), which lacked detectable levels of IgA, IgM, or IgG. This indicates that the toxin inhibitory activity was not primarily associated with immunoglobulins. Peak 1a was eluted in the void volume, in front in secretory IgA (peak b), with an apparent molecular weight of greater than 400,000. There were only minor differences in the inhibitory activity of the milk fractions against the two enterotoxins CT and LT, which are structurally and functionally very similar (reference 8 and Table 1).

When the toxin inhibitory activity was measured by ELISA, LT was markedly inhibited (Table 1). Peak 1a with the highest in vivo inhibitory activity also showed the highest LT inhibitory activity in ELISA.

In the study reported here, we have demonstrated that fractions of human milk inhibited the fluid secretion induced by CT and LT in rabbit intestines. Although the IgA fraction contained some inhibitory activity, most of the inhibitory effect of human milk was associated with a high-molecular-weight fraction depleted of secretory IgA. These results from in vivo experiments confirm our earlier in vitro studies on the LT inhibitory activity in milk (5, 7). The correlation between the LT inhibitory activity measured in ELISA and in rabbit ileal loops indicates that ELISA may be used to measure the LT inhibitory activity.

In vitro inhibitory activity of LT has been demonstrated in the 47 Norwegian milk samples so far investigated, although the concentration varied. In this study, only one of the milk samples was studied in vivo. The study will be continued with more human milk samples as well as with milk from other sources.

Although our study clearly shows that secretory IgA was not responsible for the major toxin inhibitory activity of the milk, we do not know much about the nature of this activity. We have chosen to express the inhibitory activity in relation to the total protein content of the milk fraction, but it may well be that the toxin inhibitory activity is associated with other structures. Protein structures, lipid complexes, or polysaccharides in milk may all interfere with the binding of the toxin to its receptor on the cells in the intestine or to antibody on the ELISA plate, either because the milk component(s) binds to the receptor site or antibody site of the toxin or sterically blocks these sites. Preliminary experiments showed that the inhibitory activity was extracted by chloroform and methanol, suggesting the involvement of lipid structures (7). The nature of this inhibitory activity is under current study.

This study was supported by the Norwegian Research Council for Science and the Humanities, the Anders Jahre's Fund for the Promotion of Science, Norway, and by grants from the National Defense Research Institute, Sweden (project 506-5129).

LITERATURE CITED

1. **Donta, S. T., D. A. Sack, R. B. Wallace, N. L. Dupont, and R. B. Sack.** 1974. Tissue-culture assay of the antibodies to heat-labile *Escherichia coli*. N. Engl. J. Med. **291**:117–121.
2. **Gerrard, J. W.** 1974. Breast feeding: second thoughts. Pediatrics **54**:757–764.
3. **Goldman, A. S., and C. W. Smith.** 1973. Host resistance factors in human milk. J. Pediatr. **82**:1082–1090.
4. **Hanson, L. Å., B. Carlsson, J. R. Cruz, B. Garcia, J. Holmgren, S. R. Khan, B. S. Lindblad, A.-M. Svennerholm, B. Svennerholm and J. Urrutia.** 1979. Immune response in the mammary gland, p. 145–157. *In* P. L. Ogra and D. Dayton (ed.), Immunology of breast milk. Raven Press, New York.
5. **Otnaess, A.-B., and S. Halvorsen.** 1980. Non-antibody components in human milk inhibit *Escherichia coli* heat labile enterotoxin measured by an enzyme-linked immunosorbent assay. Acta Pathol. Microbiol. Scand. Sect. C **88**:247–253.
6. **Otnaess, A.-B., and I. Ørstavik.** 1980. The effect of human milk fractions on rotavirus in relation to the secretory IgA content. Acta Pathol. Microbiol. Scand. Sect. C **88**:15–21.
7. **Otnaess, A.-B., and I. Ørstavik.** 1981. Effect of fractions of Ethiopian and Norwegian colostrum on rotavirus and *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **33**:459–466.
8. **Sack, R. B.** 1975. Human diarrhoeal disease caused by enterotoxigenic *Escherichia coli*. Annu. Rev. Microbiol. **29**:333–353.
9. **Svennerholm, A.-M.** 1975. Experimental studies on cholera immunization. IV. The antibody response to formalinized V. cholerae and purified endotoxin with special reference to protective capacity. Int. Arch. Allergy Appl. Immunol. **49**:434–452.